The mitochondrial ryanodine receptor in rat heart: A pharmaco-kinetic profile

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Abstract

A protein discovered within inner mitochondrial membranes (IMM), designated as the mitochondrial ryanodine receptor (mRyR), has been recognized recently as a modulator of Ca2+ fluxes in mitochondria. The present study provides fundamental pharmacological and electrophysiological properties of this mRyR. Rat cardiac IMM fused to lipid bilayers revealed the presence of a mitochondrial channel with gating characteristics similar to those of classical sarcoplasmic reticulum RyR (SR-RyR), but a variety of other mitochondrial channels obstructed clean recordings. Mitochondrial vesicles were thus solubilized and subjected to sucrose sedimentation to obtain mRyR-enriched fractions. Reconstitution of sucrose-purified fractions into lipid bilayers yielded Cs+-conducting, Ca2+-sensitive, large conductance (500–800 pS) channels with signature properties of SR-RyRs. Cytosolic Ca2+ increased the bursting frequency and mean open time of the channel. Micromolar concentrations of ryanodine induced the appearance of subconductance states or inhibited channel activity altogether, while Imperatoxin A (IpTxa), a specific activator of RyRs, reversibly induced the appearance of distinct subconductance states. Remarkably, the cardiac mRyR displayed a Ca2+ dependence of [3H]ryanodine binding curve similar to skeletal RyR (RyR1), not cardiac RyR (RyR2). Overall, the mRyR displayed elemental attributes that are present in single channel lipid bilayer recordings of SR-RyRs, although some exquisite differences were also noted. These results therefore provide the first direct evidence that a unique RyR occurs in mitochondrial membranes.

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1. Introduction

Extracellular Ca2+ influx through the dihydropyridine receptor induces the opening of Ca2+ release channels in cardiac muscle, giving rise to the Ca2+-induced-Ca2+ release (CICR) mechanism that triggers muscle contraction [1,2]. The Ca2+ release channel that is essential for excitation–contraction (E–C) coupling is a ∼2 million Da homotetrameric protein of the sarcoplasmic reticulum (SR) that binds ryanodine with high specificity and affinity; hence it is named the ryanodine receptor (RyR) [3–5]. A variety of endogenous substances have been described to regulate RyR activity, including Ca2+, Mg2+, ATP, H+, calmodulin, several protein kinases [6,7], and accessory proteins like sorcin and FKBP12 [8]. Likewise, several exogenous regulators of RyR activity, although not directly associated with the CICR mechanism, play an important role in defining the pharmacological profile of the RyR [9]. These modulators include: ryanodine [10], caffeine [11], dantrolene [12], and scorpion peptide toxins [13], among others. Overall, the role of the RyR in E–C coupling, its pharmacological properties, and its molecular identity are fairly well characterized.

Recently, studies have shown that an inner mitochondrial membrane (IMM) protein runs with identical mobility to SR-RyRs in SDS-PAGE, cross-reacts with RyR antibodies, and mediates ryanodine- and dantrolene-sensitive Ca2+ fluxes [14,15]. It is possible, therefore, that a mitochondrial channel protein with signature features of the RyR (i.e. a mitochondrial RyR, mRyR) may be an integral component of the Ca2+ transport system of mitochondria. If verified, this would undoubtedly be a surprising topological location for a RyR.
that would prompt the notion that there is heterogeneity of RyR localization within cardiomyocytes. In this study, we fused purified mitochondrial membranes into planar lipid bilayers and determined the existence of a mitochondrial channel that behaves remarkably similar to SR-RyRs. We show that the unitary channel conductance and open probability of this presumed mRyR in the presence of some of the most classical modulators of RyR activity, including: Ca\(^{2+}\), ryanodine, and Imperatoxin A (IpTxA, is a 3.7 kDa peptide toxin, isolated from the venom of the scorpion Pandinus imperator, with high affinity to and exquisite selectivity for RyRs) are remarkably similar to those of the SR-RyR. However, a unique identifying feature of this mRyR was its Ca\(^{2+}\)-dependence of activity curve, which was similar to that of skeletal RyR (RyR1), instead of that of cardiac RyR (RyR2), as would be expected from its tissue localization. These results were in concordance with those of Beutner et al. [14,15] and reinforce the notion that a mRyR is an integral component of mitochondrial membranes and not a residual component of SR. Several studies support the idea that mitochondria sequester significant amounts of mobilized cytosolic Ca\(^{2+}\) in cardiac muscle cells, thereby shaping cytosolic Ca\(^{2+}\) transients [16–21], and also controlling cellular energy production on a beat-by-beat basis [22]. Thus, a potential role of the mRyR may be to modulate Ca\(^{2+}\) fluxes in the mitochondria through Ca\(^{2+}\)-induced Ca\(^{2+}\) uptake, perhaps simultaneously functioning as a transducer for excitation–metabolism coupling in cardiac cells [15].

2. Materials and methods

2.1. Materials

[\(^{1}\)H]Hydantoin was purchased from NEN Life Science Products (Shelton, CT). The antibody against the voltage-dependent anion channel (VDAC) was obtained from Calbiochem (San Diego, CA) and the antibodies against the sarco- and endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA2) and calsequestrin were from Santa Cruz Biotechnology (Santa Cruz, CA). Porcine brain phosphatidylethanolamine and phosphatidylserine were purchased from Avanti Polar Lipids (Birmingham, AL). Precast SDS-polyacrylamide gels were from Bio-Rad (Hercules, CA). All other chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted.

2.2. Methods

2.2.1. Isolation of rat heart mitochondria

Heart mitochondria were isolated in isotonic ice-cold mannitol/sucrose buffer (M/S buffer; in mM: 225 mannitol, 75 sucrose, 0.5 EGTA, 1 glutathione, 10 HEPES, pH 7.4) by differential centrifugation and subsequent purification on a Percoll gradient as described previously [23].

2.2.2. Preparation of mitochondrial subfractions

Mitochondrial subfractions were prepared as described previously [14,24]. Isolated mitochondria from rat hearts were osmotically shocked in 10 mM Na\(_2\)HPO\(_4\)/NaH\(_2\)PO\(_4\) for 20 min on ice followed by the addition of sucrose (20% final concentration). Mitochondrial membranes were disrupted by sonication (two times for 30 s), and eventually intact mitochondria were removed by centrifugation at 7000×g for 20 min. The supernatant was transferred as a final layer onto a continuous sucrose gradient (from 60% to 30% sucrose in 10 mM HEPES, pH 7.4, plus protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN)) and centrifuged for at least 8 h at 70,000×g to separate mitochondrial membrane vesicles. The mitochondrial subfractions were tested for specific marker proteins: succinate dehydrogenase (SDH) for IMM (detailed below), creatine kinase for contact sites (CS) between IMM and outer mitochondrial membrane (OMM), and the VDAC for OMM. The characterized CS and IMM fractions were diluted 2-fold with M/S buffer, which contained protease inhibitor cocktail, and centrifuged for 90 min at 300,000×g to sediment the mRyR. The pellet was resuspended in a small volume of M/S buffer and was stored in liquid nitrogen until needed. The IMM samples used for these experiments tested negative for SERCA2, calsequestrin, and VDAC (marker proteins for the SR and OMM, respectively). Likewise, the CS samples tested negative for SERCA, but were slightly contaminated with VDAC.

2.2.2.1. Succinate dehydrogenase test

Succinate dehydrogenase (SDH) was measured enzymatically according to the procedure of Pette [25]. Two to 5 µl sample volume (for IMM) or 50 to 100 µl sample volume (for other mitochondrial subfractions) was added to an assay mixture containing (in mM): 1 sodium phosphate, 1 potassium cyanide, 0.1 phenazine methosulfate and 0.1 cytochrome c. The reaction was started with 10 mM succinate. The increase of absorbance was measured with a Genesis 5 spectrophotometer at 550 nm.

2.2.2.2. Denaturing SDS-PAGE and Western blot analysis

For the determination of SERCA and calsequestrin contamination within the mitochondrial preparations, aliquots (50 µg and 30 µg, respectively) of mitochondrial subfractions were loaded in 10% SDS-polyacrylamide gels. The separated proteins were transferred onto nitrocellulose membranes for 90 min at 100 V. Western blots were performed using the Amersham enhanced chemiluminescence assay. To determine the validity of the RyR purifications (described below), some of the collected fractions were stained with silver salts by modifications of a previously described procedure [26]. Briefly, 40 µg of protein was subjected to SDS-polyacrylamide gel electrophoresis in a 7.5% polyacrylamide precast mini-gel. The gel was incubated for 1 h in 50 ml of fixing solution (ethanol: acetic acid: water; 15:5:30). After discarding the fixing solution, the gel was incubated twice in 50 ml of 30% ethanol for 30 min with gentle shaking. The gel was washed 3 times, 10 min each, in 100 ml of water and then incubated for 30 min in a silver solution (AgNO\(_3\)/H\(_2\)O\(_2\); 1:20). After washing with deionized water, the gel was treated with 50 ml of an aqueous solution containing 2.5% sodium carbonate and 0.02% formaldehyde until stained bands were apparent. The reaction was quenched by washing the gel in 1% acetic acid for a few minutes followed by washing with deionized water.

2.2.2.3. Preparation of SR-enriched microsomes from skeletal and cardiac muscle

Skeletal SR was isolated from frog hind leg muscles and cardiac SR was isolated from rat hearts by differential centrifugation as described [27,28]. Briefly, dissected muscle was placed in ice-cold saline solution (0.9% NaCl, 10 mM Tris–HCl, pH 6.8), minced to small pieces, weighed, and homogenized with 3 volumes of saline solution in a Waring blender for 2 min at high speed. The saline solution contained the following protease inhibitors: leupeptin (12 µM), phenylmethylsulphonyl fluoride (PMSF; 100 µM), benzamidine (10 µM), and aprotinin (10 µM). The blended tissue was homogenized more thoroughly using a Brickman Polytron (three times for 15 s each on setting 2). The homogenate was centrifuged at 4000×g for 20 min, and the resultant supernatant was filtered through 4 layers of cheese cloth and centrifuged again at 8000×g for 20 min. The supernatant from the 8000×g spin was centrifuged at 40,000×g for 30 min. The 40,000×g pellet, containing the SR-microsomes, was resuspended in a sucrose buffer (containing 0.3 M sucrose, 0.1 M KCl, 5 mM Na-PIPES, pH 6.8), aliquoted, quickly frozen, and stored at −80 °C before use.

2.2.2.4. Purification of SR-RyR and mRyR

The frog skeletal microsomes and isolated IMM and CS subfractions were subsequently purified via sucrose gradient centrifugation [5,29]. The thawed samples were diluted 1:1 with solubilization buffer (final concentrations: 1 M NaCl, 40 mM Na-PIPES, 100 µM CaCl\(_2\), 0.5% CHAPS, 1:5000 dilution of protease inhibitor cocktail (Sigma), pH 7.2) and incubated for 1 h on ice. The solubilized preparations were sedimented by centrifugation at 4 °C for 40 min in...
a Beckman Ti45 rotor at 100,000×g. The RyR-containing supernatant from the solubilized samples was layered on top of 32 ml continuous sucrose gradients (containing 5–20% sucrose, 0.3 M NaCl, 40 mM Na-PiPES, 300 μM CaC12, 0.1% CHAPS, pH 7.5). Following centrifugation in a Beckman SW28 rotor for at least 16 h at 76,000×g at 4 °C, fractions were collected (automatic pump set at 1.5 ml/tube) from the bottom of the tube upwards (with fraction #1 containing 20% sucrose and the final fraction containing 5% sucrose). This fractionation resulted in 20 tubes per gradient, and each fraction was silver-stained (see above) and corresponded to the luminal side of the channel. In this configuration, Cs+ RyRs were added to the cis (cytosolic) side of the chamber, which contained the methanesulfonate, 20 mM NaCl, 20 mM KCl added to both sides of the chamber in order to stimulate channel flows from the luminal to the cytosolic side at negative holding potentials. Cs+ did not correspond to SR channels.

2.2.7. [3H]ryanodine binding assay

[3H]ryanodine binding to mitochondrial membrane vesicles was carried out as described previously [14]. Briefly, 100 μg of rat heart mitochondrial membranes were incubated in 0.17 M KCl, 10 mM MOPS (pH 7.0), 10 nM [3H]ryanodine, and with various concentrations of CaCl2 to set free Ca2+ concentration in the range of 10 nM to 1 mM (total volume 500 μl). The incubation occurred in the presence (+IpTxa) or absence (control) of 1 μM IpTxa, and lasted for 4 h at 37 °C. Likewise, [3H]ryanodine binding to rat cardiac SR was carried out as described previously [32]. Briefly, the standard incubation medium contained 0.2 M KCl, 30 mM MOPS (pH 6.8), 100 μg of cardiac microsomes, 10 nM [3H]ryanodine, 1 mM EGTA, and CaCl2 necessary to set free concentration of Ca2+ in the range of 10 nM to 10 mM (total volume 100 μl). The incubation occurred in the presence (+IpTxa) or absence (control) of 1 μM IpTxa, and lasted 90 min at 37 °C. Samples were always run in duplicate, filtered onto glass fiber filters (Whatman GF/C), and washed three times with 5 ml of cold water using a Brandel M-24R cell harvester (Gaithersburg, MD). The filters were placed in scintillation vials, 7 ml of liquid scintillation mixture was added, and the retained radioactivity was measured in a Beckman LS-6500 β-counter. The specific binding was defined as the difference between the binding in the absence (total binding) and presence (nonspecific binding) of 10 μM unlabeled ryanodine.

2.2.8. Planar bilayer recordings of RyRs

Our single channel recordings in lipid bilayers were performed as described previously for the SR-RyR [32,33]. The mRyRs or purified frog skeletal RyRs were reconstituted into planar lipid bilayers composed of phosphatidylethanolamine and phosphatidylserine (1:1) dissolved in decane at a concentration of 20 mg/ml. Single channel data were collected at steady negative voltages (which varied depending on the experiment) in symmetrical 300 mM cesium (Cs+) methanesulfonate, 20 mM Na-HEPES, pH 7.2. Proteoliposomes or purified RyRs were added to the cis (cytosolic) side of the chamber, which contained the voltage command electrode. The trans chamber contained the reference electrode and corresponded to the luminal side of the channel. In this configuration, Cs+ flows from the luminal to the cytosolic side at negative holding potentials. Cs+ was chosen as the charge carrier to increase the channel conductance and to avoid interference from K+ channels present in the SR and inner mitochondrial membranes. Chloride channels were mostly blocked by replacing chloride with the impermeant methanesulfonate anion. Contaminant Ca2+ (∼3–5 μM, as determined by a Ca2+ electrode) served to activate RyRs. In some experiments, 50 μM CaCl2, 10 μM ryanodine, or 500 nM IpTxa was added to the cis side of the channel, as detailed in the text. Some of the recordings were also made with 20 mM KCl added to both sides of the chamber in order to stimulate channel activity [34], as specified in the text. Channel activity was filtered with an 8-pole low pass Bessel filter set at 1.5 kHz and digitized at a rate of 4 kHz using a Digidata 1200 AD/DA interface. Data acquisition and analysis were performed with Axon Instruments (Burlingame, CA) hardware and software (pClamp 8).

3. Results

3.1. The mRyR is distinct from the SR-RyR

Calsequestrin and SERCA are specific marker proteins for the SR. To determine if the rat heart mitochondrial subfractions had any SR contamination, Western blots performed with an antibody against the cardiac isoform of SERCA (SERCA2a) showed that the rat cardiac SR-containing cytosol (Fig. 1A, lane 1), but not mitochondrial subfractions (Fig. 1A, lanes 2–6) had immunoreactivity against this antibody. Likewise, cardiac SR and mitochondrial vesicles from rat hearts retained the presence of calsequestrin (Fig. 1B, lanes 1–2); however, further purification and separation of mitochondrial vesicles into OMM, CS, and IMM completely removed calsequestrin from these preparations. All IMM fractions were free of the VDAC, a marker protein for the OMM (not shown, see [14,15]). The CS fraction, consisting of attachments of the OMM and IMM, was mostly SERCA2a-free (Fig. 1A, lane 3). To further ensure the quality of the IMM fraction, we measured the activity of succinate dehydrogenase, an IMM-resident enzyme. During the purification of IMM vesicles the activity of this enzyme should increase because matrix proteins or proteins of the OMM are removed. As indicated in Table 1, the specific activity for succinate dehydrogenase increased from 1.35±1.06 μM/mg protein in the OMM to 2598.6±318.6 μM/mg protein in the IMM. We used IMM vesicles in most lipid bilayer experiments after verifying the lack of detection of OMM and SR proteins. Thus, we can safely ascertain that the channels we labeled “mRyR” in this study are localized in the mitochondria and do not correspond to SR channels.

To further demonstrate that the IMM fractions were not contaminated with SR components, we conducted Ca2+-dependence of [3H]ryanodine binding curves to IMM and SR vesicles from rat hearts. The rationale was based on the surprising finding that the mRyR cross-reacts with an anti-RyR1 (skeletal isoform) antibody, not an anti-RyR2 antibody [15]. Further, if IMM contained RyRs from SR vesicles, then both preparations would show a RyR2-like response to Ca2+, which differs from a RyR1-like response in the modest Ca2+-dependent inactivation occurring at high [Ca2+]. Finally, we conducted these experiments in the absence and the presence of IpTxa, a high-affinity peptide ligand of RyRs [32,35] that affects differently the Ca2+-dependence of [3H]ryanodine binding curve to RyR1 and RyR2 channels. IpTxa increases [3H]ryanodine binding to RyR1 at all [Ca2+], but it produces dual effect on RyR2, initially increasing (pCa 7–5) and then decreasing (pCa 4–2) [3H]ryanodine binding. The result is in an augmented bell-shaped curve in RyR1 and a sigmoidal curve in RyR2 [32,35]. Thus, IpTxa could exaggerate RyR isofrom-dependent differences in [3H]ryanodine binding, which could help estimate the level of SR contamination in our mitochondrial membranes. Fig. 1C shows the Ca2+-dependence of [3H]ryanodine binding to the isolated cardiac mitochondrial vesicles and the effect of IpTxa. Specific binding in the absence of IpTxa (control) had a threshold for detection at ∼100 nM Ca2+ (pCa 7) and was maximal at 10 μM Ca2+ (pCa 5). Higher Ca2+ concentrations decreased [3H]ryanodine binding, resulting in a bell-shaped curve remarkably similar to that of RyR1 [32,35]. In the presence of IpTxa (+IpTxa) the binding curve was also bell-shaped but significantly augmented by ∼2-fold (see figure legend). By contrast, the Ca2+-dependence of [3H]ryanodine binding to SR vesicles displayed a different profile (Fig. 1D,
control) and was unevenly affected by IpTxa (+IpTxa). In the most extreme example, that of the Ca\(^{2+}\)-dependent inactivation of binding at pCa 3 in control conditions, binding is almost null in RyR1\(^{32,35}\) and mRyR (Fig. 1C), but robust in cardiac SR (Fig. 1D). Thus, mRyR behaved like RyR1 in \(^{3}H\)ryanodine binding assays, and not as RyR2 as expected if the mitochondrial samples used in this study were contaminated by SR proteins. Our results therefore suggest that the mRyR isolated from mitochondrial membrane vesicles indeed originates from a separate pool of RyRs.

3.2. Diversity of ion channels in mitochondrial vesicles

In an attempt to characterize the single channel kinetics of the mRyR, we reconstituted rat IMM or CS vesicles into planar lipid bilayers as described\(^{32,33}\). The conditions used for these experiments (see Materials and methods) theoretically should favor recording of large currents through RyRs while silencing other unrelated channels. In some experiments, 20 mM KCl was added to the recording solution, as Cl\(^-\) ions have proved effective in enhancing RyR activity\(^{34}\). A variety of substates from individual or multiple types of channels were generally seen, obstructing the identification of unitary conductances (Fig. 2A, B), but even then, there were instances in which a potential mRyR was conspicuous within the recording. For example, the expanded section of the recording contained single channel fluctuations that had gating kinetics and unitary conductance remarkably similar to SR-RyR. The full, unitary Cs\(^+\) conductance of this 500 ms segment was 661 pS; in addition, the channel was unresponsive to 2 \(\mu\)M CsA, a concentration that effectively blocks the mitochondrial permeability transition pore (PTP)\(^{36}\). These brief occurrences of RyR-like channel gating were promising and demonstrated the possibility that, in the absence of other unrelated currents, a channel from the IMM with similar conductance as the SR-RyR may fuse into the planar lipid bilayer.

Given the heterogeneity of channels in the mitochondrial membranes\(^{37,38}\), other channels could be able to conduct Cs\(^+\) as well and may have been enhanced by the presence of KCl in the recording medium. Fig. 2B shows traces of steady-state activity from several channels or multiple substates of an

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<tr>
<th>Compartment</th>
<th>SDH activity [mU/mg]±SE</th>
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<tr>
<td>Mitochondria</td>
<td>704.837±68.351</td>
</tr>
<tr>
<td>Cytosol</td>
<td>10.138±2.364</td>
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<tr>
<td>OMM</td>
<td>1.355±1.062</td>
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<tr>
<td>CS</td>
<td>337.629±55.646</td>
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<tr>
<td>IMM</td>
<td>2598.589±318.596</td>
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individual channel recorded from IMM vesicles at a holding potential of \(-30\) to \(-40\) mV in the presence of nominal levels of Ca\(^{2+}\) (\(\sim 3–5\) μM). Interestingly, several mitochondrial currents recorded such as traces 2B-b, c, and e, had extremely fast gating and transitioned between a variety of conductance states. These channels/channel substates most likely correspond to the PTP and/or VDAC, or translocases of the inner membrane, when compared with published electrophysiological characterizations of these channels (for review see [36]). Trace 2B-c, for example, was inhibited by the addition of 2 μM CsA as shown in Fig. 2 (see the last 1.2 s) suggesting that this channel is indeed the PTP.

### 3.3. Purification of mitochondrial subfractions by sucrose gradient centrifugation

Single channel recordings such as those depicted in Fig. 2 substantiated the necessity for additional purification of the mitochondrial subfractions in order to isolate the mRyR. We used sucrose-gradient sedimentation of solubilized IMM or CS vesicles to purify the mRyR, as originally described for SR-RyR [5,29]. The sedimentation coefficient for the RyR is 30 S [39], thus the RyR localizes in the heavier sucrose fractions with this technique. Frog skeletal and pig cardiac SR-enriched microsomes were subjected to the same treatment as the mitochondrial subfractions and were used as positive controls (Fig. 3, Frog-F1 and Pig-F1). The occurrence of a high molecular weight band (\(\sim 500\) kDa) in the purified IMM or CS fractions with migration similar to SR-RyR monomers was confirmed by silver staining (Fig. 3). Fig. 3 verifies that the mRyR localized within the heavier, 20% sucrose fractions (IMM-F1, CS-F3). The detection of the mRyR within the heavier sucrose fractions confirmed that the mRyR may be isolated in an analogous...
manner as the SR-RyR; we thus obtained a more purified preparation to use in lipid bilayer experiments.

3.4. Single channel kinetics of the mRyR

In order to facilitate fusion of mRyR into planar lipid bilayers, we reconstituted the solubilized IMM and CS proteins into proteoliposomes using the procedure described by Lee et al. [31]. We used single channel conductance as the first of a series of biophysical and pharmacological criteria to designate mitochondrial channels as mRyRs [40,41]. Accordingly, profiles of single channels likely corresponding to mRyRs solely based on their unitary channel conductances were recorded (n=21). Fig. 4A shows six representative channels reconstituted from the IMM or CS purified fractions that display conductance values similar to Cs⁺-conducting SR-RyRs. Traces a, b, and c represent channels fused from purified IMM proteoliposomes, while traces d, e, and f are channels fused from purified CS proteoliposomes. All channels depicted in Fig. 4A were recorded at −20 to −50 mV, thus downward deflections of the baseline current correspond to channel openings. In all traces there are discrete, fully-resolved openings that are characteristic of classical RyRs. Some of the openings are longer lasting (traces c and d) and some are fast-flickering (trace f). In some of the recordings (traces a and e), the channels enter into periods of half-conductance states that are reminiscent of the intrinsic subconductance states present in SR-RyRs [39]. Thus, most kinetic states previously reported for SR-RyRs [7] are represented in our mRyR recordings. Fig. 4B displays a representative single channel current–voltage relationship of these mitochondrial channels (trace 4A-a). In the range of −30 to +20 mV, the slope conductance was 616±41 pS when measured in symmetrical 300 mM cesium methanesulfonate solution. Overall, the unitary conductance for the purified IMM or CS channels presented in Fig. 4 fluctuated between 550 and 790 pS, values which are close to those expected for SR-RyR under these recording conditions [42]. The slight but significant deviation of these values from the average Cs⁺-conducting SR-RyR is most likely due to modifications of the recording solution (See Discussion).

3.5. Calcium activation of the mRyR

Next, we investigated the effects of some classical RyR modulators on mitochondrial channels that had conductance measurements appropriate for RyRs as described above. The RyRs in the SR of both cardiac and skeletal muscle are activated by nanomolar to micromolar concentrations of Ca²⁺ [34]. We...
thus aimed to determine whether Ca\(^{2+}\) has a similar effect on the mRyR. For a side-by-side comparison of solubilized and partially purified channels, we used the RyRs from frog skeletal muscle that were separately subjected to the sucrose sedimentation technique described in Fig. 3. Frog skeletal muscle is highly enriched with RyRs, and although it contains two RyR isoforms (alpha-RyR and beta-RyR, [43]) they are kinetically indistinguishable from mammalian RyRs in single channel recordings, thus we chose this high-density RyR preparation as our control. Fig. 5 shows that the frog skeletal SR-RyR and the rat cardiac mRyR response to Ca\(^{2+}\) was strikingly similar. The top single channel recordings of the SR-RyR and mRyR (Fig. 5A, B, respectively) represent control conditions in the absence of modulators except for the presence of nominal levels of Ca\(^{2+}\) (\(~3–5\, \mu M\)). Under these control conditions, the conductance for the SR-RyR and the mRyR was 555 and 626 pS, respectively. Ca\(^{2+}\) (50 \(\mu M\) added to the cis chamber) activated the SR-RyR and mRyR by increasing both bursting frequency and mean open time (Fig. 5C, D). Following addition of Ca\(^{2+}\), the open probability \((P_o)\) increased from 0.065 to 0.36 and from 0.034 to 0.40 for the SR-RyR and the mRyR channel, respectively. A more quantitative description of the effect of Ca\(^{2+}\) on channel activity can be calculated from the open time histograms (Fig. 5G, H). Under control conditions, open events of the SR-RyR could be fit best by a double exponential with \(\tau_{\text{open1}}=0.979\pm0.047\, \text{ms (61%)}\) and \(\tau_{\text{open2}}=5.453\pm0.029\, \text{ms (39%)}\), while mRyR openings were best fit with a single exponential, \(\tau_{\text{open}}=0.662\pm0.049\, \text{ms}\). In the presence of 50 \(\mu M\) Ca\(^{2+}\), the open time histograms were biexponential for both SR-RyR \([\tau_{\text{open1}}=0.746\pm0.056\, \text{ms (55%)}\) and \(\tau_{\text{open2}}=4.621\pm0.016\, \text{ms (45%)}\]) and the mRyR \([\tau_{\text{open1}}=0.597\pm0.153\, \text{ms (53%)}\) and \(\tau_{\text{open2}}=3.593\pm0.034\, \text{ms (47%)}\]). These data (Fig. 5G and H) and the current amplitude histograms for the SR-RyR and the mRyR (Fig. 5E and F, respectively) delineate the agonistic effect of Ca\(^{2+}\) on both groups of channels.

3.6. Ryanodine activation of the mRyR

In the presence of sub- to low micromolar concentrations of ryanodine, SR-RyRs lock into a distinct conformational state

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**Fig. 5.** Comparison of the Ca\(^{2+}\) activating effect on SR-RyR and the mRyR. Single channel recordings of purified RyRs in planar lipid bilayers composed of phosphatidylethanolamine and phosphatidylserine (1:1). Single channel data were collected at steady voltages (−25 to −30 mV) in symmetrical 300 mM Cs\(^+\) methanesulfonate after dissipation of the 50 mM Cs\(^+\) methanesulfonate gradient in the trans chamber. Proteoliposomes of purified RyRs were added to the cis side of the channel, which contained the voltage command electrode. (A) Representative recordings of the baseline activity of a RyR channel from frog skeletal muscle (SR-RyR) and (B) the IMM (mRyR) from rat cardiac preparations in the absence of added Ca\(^{2+}\). (C) Ca\(^{2+}\) increases the open probability \((P_o)\) of channel activity of the SR-RyR from frog skeletal muscle and (D) the rat cardiac IMM \((n=5)\). (E and F) Current amplitude histograms of RyR channel activity from frog skeletal muscle and the rat cardiac IMM, respectively, before and after the addition of Ca\(^{2+}\). (G and H) Mean open time histograms of the frog skeletal SR-RyR (G) and the rat cardiac mRyR (H) before (black bars, Control) and after (gray bars) the addition of Ca\(^{2+}\). The light gray and black solid lines represent the exponential decay fittings of the dwell time plots before and after the addition of Ca\(^{2+}\), respectively. The number of observations collected during a 30 s period were 695 (SR-RyR Control), 1454 (SR-RyR with Ca\(^{2+}\)), 335 (mRyR Control), and 2059 (mRyR with Ca\(^{2+}\)). c: closed state of the SR-RyR and mRyR. * indicates the number of open events before and after the addition of Ca\(^{2+}\) for the SR-RyR and the mRyR.
characterized by long-lasting subconducting openings representing ~50% of the full conductance [3]. We therefore tested the effect of ryanodine on mitochondrial channels. Fig. 6 shows an IMM channel reconstituted in a lipid bilayer in the absence (control, Fig. 6A-a), and the presence (Fig. 6B-a) of 10 μM ryanodine added to the cis chamber. Under control conditions (Fig. 6A) the channel displayed quick, intermittent openings that resulted in overall low $P_o$ of 0.023 for the duration of the recording. The diary of activity for the first 20 s of channel recording (Fig. 6A-b) emphasizes the quiescent nature of this channel in the absence of external modulators. By contrast, addition of 10 μM ryanodine to the channel (Fig. 6B) dramatically altered its activity by inducing the appearance of a half-conducting state that lasted for several seconds as depicted in the diary of openings (Fig. 6B-b) and the current amplitude histogram (Fig. 6B-c). Even then, the channel also showed an uncharacteristic response to ryanodine by alternating between periods of sub- and full-conducting states for the first ten s, followed by complete inhibition throughout the remainder of the recording (Fig. 6B-b). As a result of this bimodal effect, the $P_o$ initially increased to 0.97, presumably while ryanodine exerted its activating effect, and then fell to zero as ryanodine exerted a blocking effect. The normalized $P_o$ for the first 20 s was therefore 0.13 and zero thereafter. Interestingly, we found no mitochondrial channels that irreversibly locked into subconducting states by ryanodine. There were channels completely inhibited by 10 μM ryanodine ($n=2$), while other channels displayed alternating periods of sub- and full-conducting states ($n=3$) in response to ryanodine.

### 3.7. Imperatoxin A induces mRyR subconductance

Unlike its differential effects on the Ca$^{2+}$ dependence of [3H]ryanodine binding, IpTxa affects the single channel kinetics of all RyR isoforms (RyR1, RyR2 and RyR3) in a similar manner [35,44]. Like ryanodine, IpTxa at nanomolar concentrations induces the appearance of long-lasting subconductance states in both SR-RyR isoforms, RyR1 and RyR2. At negative recording potentials (such as those used for these experiments), the substate conductance represents approximately 43% of the full conductance openings [35]. Unlike ryanodine, however, the reversible binding of IpTxa allows the channel to fluctuate between sub- and full-conducting states. Fig. 7 shows that, remarkably, IpTxa exhibited its classical activating effect on a reconstituted mRyR channel purified from mitochondrial subfractions.

The mRyR depicted in Fig. 7A was recorded under control conditions (in a nominally-free Ca$^{2+}$ solution without additional modulators) and had gating kinetics characteristic of SR-RyRs, i.e., numerous fast openings alternating with few long-lasting...
openings, both giving rise to sporadic bursts of channel activity. The overall activity and conductance of the channel are summarized in the current amplitude histogram (Fig. 7A, right panel). Conversely, the same channel after addition of 500 nM IpTxₐ (Fig. 7B, left panel) and the resulting amplitude histogram (Fig. 7B, right panel) displayed the signature effect of IpTxₐ on the mRyR. The toxin induced long-lasting subconducting states interspersed between periods of full-
conducting channel activity. As expected, the substate conductance approximated 40% of the full conductance openings. The amplitude histogram of mRyR channel activity (Fig. 7B, right panel) emphasizes that modulation by IpTxa increased the $P_o$ of the mRyR by increasing the frequency of channel openings and decreasing the duration of the closed events. Indeed, mRyR $P_o$, augmented from 0.13 in control to 0.86 in the presence of IpTxa (Fig. 7A and B, respectively). In Fig. 7C we compared the gating of the presumed mRyR with that of frog SR-RyR recorded under identical experimental conditions. As shown in the compressed and expanded traces, the activity of both channels oscillated between periods of long-lasting subconductance states, representing occupation of the channel by IpTxa [35], and normal full conductance openings, representing gating of IpTxa-free channels. Notice the remarkable resemblance in the gating characteristics of both channels.

4. Discussion

The availability of techniques that segregate mitochondrial subfractions from SR and other membrane particulates [14,15] has made it possible to identify a channel protein with physical characteristics similar to those of SR-RyRs in an unusual location, the mitochondria. The differential effects of IpTxa on the Ca$^{2+}$ dependence of $[^3]$Hryanodine binding (Fig. 1C, D) reinforce the notion that mRyR has exquisite functional differences with the SR-RyR from cardiac tissue. Likewise, Western blots of the mitochondrial subfractions recognize a high molecular weight (~500 kDa) band within the IMM that cross-reacts with RyR1 (skeletal isofrom) but not RyR2 (cardiac isofrom) specific antibodies, again strengthening the assertion that this protein is a unique mitochondrial component and not an SR contaminant (which would otherwise yield a strong RyR2 signal) [15]. $[^3]$Hryanodine, a specific ligand of the SR-RyR, binds to mitochondrial vesicles with high affinity, and in the presence of ryanodine, Ca$^{2+}$ uptake into isolated heart mitochondria is substantially suppressed [14]. Thus, a protein similar to SR-RyR appears to be an important functional Ca$^{2+}$ transporter within the IMM.

The present study demonstrates that the mitochondria contain a functional RyR within the IMM that shares several pharmacological and electrophysiological characteristics with the SR-RyR at the single channel level. We reasoned that the presently described mRyR is of mitochondrial origin and free from RyR2 contamination on the following grounds: (1) IMM and CS subfractions were free from detectable amounts of SERCA and calsequestrin protein (Fig. 1A and B, respectively), indicative for SR; (2) some of the channels fused into lipid bilayers were blocked by CsA, indicating that these vesicles contained the mitochondrial PTP; (3) ryanodine exerted distinctive effects on mRyR that differ from those exerted by the alkaloid on RyR2 (Fig. 6), suggesting that mRyR represents a RyR isoform that retains the bulk of RyR properties but maintains unique characteristics, akin to the different Ca$^{2+}$-dependence of channel activity seen with RyR1 and RyR2 [34,45]; and (4) the Ca$^{2+}$ dependence of $[^3]$Hryanodine binding in the presence and absence of IpTxa to IMM vesicles was analogous to that expected for RyR1 (Fig. 1C, D), consistent with Western blot data [15], and different from that of RyR2, as would be expected if cardiac SR vesicles contaminated our IMM preparations.

The IMM contains a variety of proteins, which exhibit channel activity in bilayer membranes, including the Ca$^{2+}$ uniporter [46], the ANT [47–49], and proteins of the mitochondrial protein transport system [50]. Several studies support the hypothesis that the PTP is a protein complex containing components of the IMM, such as the ANT, and the OMM, such as VDAC [51,52]. Given the high sensitivity of our electrophysiological assay, it was not surprising that we reconstituted a multiplicity of ion channels from the IMM or CS subfractions (Fig. 2). Some of the traces depicted in Fig. 2 were from channels of unknown molecular identity, so it is likely that we recorded IMM transport proteins that have not yet been fully characterized in lipid bilayer experiments. Unfortunately, our single channel lipid bilayer assay does not allow us to determine the precise location of the mRyR within the distinct mitochondrial substructures. However, VDAC, a marker protein for the OMM, was below detection levels in Western blot characterization of IMM vesicles, which most likely excludes the localization of mRyR in OMM. Importantly, we had the most success fusing mRyR channels from the IMM preparations (the mitochondrial subfracton used for the majority of the experiments described in this study). Therefore, if we make the reasonable assumption that the likelihood of detecting a protein in a given vesicular preparation is directly proportional to the relative abundance of the protein in the same preparation, then the higher frequency of mRyR detection in IMM vesicles favors the notion that IMM is the preferred location of this channel, in agreement with supporting data obtained by electron microscopy and Western blot analysis with RyR antibodies [14,15].

Traditionally, the enormous mass (~2 million Da/channel tetramer) and sedimentation coefficient (30 S) of the SR-RyR have been exploited for separation of this channel from other SR proteins [29,39]. Purification by sucrose gradient centrifugation also proved successful to separate the mRyR from other mitochondrial channels (Fig. 3). This provided further evidence that the mRyR is a channel complex that can be purified with the same methods used for the SR-RyR. Nevertheless, in spite of mRyR enrichment, we continued to detect “contaminating”
channels from purified subfractions that did not appear to correspond to the mRyR. The predominant types of unrelated channels were blocked by CsA, suggesting that the PTP may have co-segregated with the mRyR in our sucrose sedimentation procedure. It has been hypothesized that the PTP is a multi-protein complex, which may consist of VDAC, the ANT, hexokinases [51], and the benzodiazepine receptor [53] all having a total molecular weight of ~600 kDa. Given the large molecular weight of the PTP multi-protein complex and that of the mRyR, it is probable that both of these protein complexes could colocalize within the heavier sucrose fractions.

In order to characterize the mRyR at the single channel level, we established a set of criteria to designate certain channels as mRyR candidates. The first index of activity to categorize a mitochondrial channel as potential mRyR was single channel mRyR candidates. Smith et al. [42] found that RyR1 conducts Cs⁺ at higher rate than other monovalent cations. For example, in recording solution containing 50 mM cis and 250 mM trans [Cs⁺], the unitary conductance of RyR1 is ~500 pS [42]. For the present study, we used 50 mM cis and 300 mM trans [Cs⁺] (and then dissipated the gradient following channel fusion so that both sides of the mRyR contained 300 mM Cs⁺), predicting mRyR single channel unitary conductances of 500–600 pS. Nevertheless, the addition of KCl to the recording solution may have caused the mRyR conductance to deviate from those values expected for a Cs⁺-conducting RyR1. When millimolar concentrations of KCl were added to the recording solution to favor channel activity, we expected an increase of RyR conductance to above 600 pS [42], which we observed in the single channel mRyR experiments recorded under these conditions (Fig. 4A, traces a–e). This was possibly the single most significant factor in the unitary conductance variation we observed (Fig. 4), followed perhaps by Cs⁺ leak from cis to trans chambers during bilayer re-painting and the intrinsic noise of the high-frequency recording bandwidth (1.5 kHz).

The second criterion to designate channel activity as that corresponding to mRyR was the lack of response to CsA (~2 μM), excluding the possibility that the channel recordings represented substates of the PTP, which can rapidly flicker from its fully closed state to a substate of 600–700 pS [37]. Finally, we expected the candidate channels to be sensitive to Ca²⁺, ryanodine, and IpTx₃ (Figs. 5–7) because, although with small variations, these modulators produce signature effects on all RyR isoforms. The profile of the single channel recordings and the unitary Cs⁺ conductance measurements of the channels depicted in Fig. 4A, as well as their lack of modulation by CsA, confirmed that these channels are indeed representative of the mRyR. Likewise, as seen in Figs. 5–7, the mRyR displayed the elemental attributes that are present in single channel lipid bilayer recordings of a SR-RyR and responded similarly to classical modulators of SR-RyR activity.

Using these criteria, we have determined some of the most salient pharmacokinetic characteristics of the mRyR. The mRyR described in this study displays gating properties reminiscent of those seen in the classical SR-RyR, including an analogous conductance for monovalent cations and Ca²⁺ sensitivity. The mRyR conforms to a ryanodine-altered substate in the presence of the ligand, and IpTx₃ binds reversibly to the mRyR allowing for the oscillation of channel activity from subconducting to full-conducting states. Future experiments should determine whether the typical SR-RyR accessory proteins are associated with this intriguing mRyR and if their modulatory effects are similar in a novel environment. Equally important, the present functional assays should provide a practical platform to test the role of the mRyR as a modulator of Ca²⁺ fluxes in mitochondria and as a transducer for excitation–metabolism coupling in cardiac cells.

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